

**REMARKS**

**Summary of Status of Amendments**

Upon entry of this amendment, claims 1 and 6 will be amended. Claims 1-9 are all the claims pending in this application.

Applicants note that the minor amendment to claims 1 and 6 were made to more clearly recite the claimed subject matter. Claim 1 has been amended to include, amongst other features, “mesenchymal”. Support for the amendment to claim 1 may be found throughout the original specification, for example, at page 3, lines 1-4 and page 7, line 15. Claim 6 has been amended to even more clearly recite “a 5 cm diameter RWV vessel is used” as suggested by the Office Action. Support for the amendment to claim 6 may also be found, for example, at page 7, line 6 and Figure 2.

No new matter is added.

**Information Disclosure Statement and Drawings**

Applicants thank the Examiner for acknowledgement and acceptance of the drawings filed June 7, 2006.

Applicants thank the Examiner for acknowledgement of receipt of the Information Disclosure Statement filed June 7, 2006, by returning an initialed copy of the Form PTO SB/08 therein.

**Claim to Priority**

Applicants thank the Examiner for acknowledgement of the claim to priority to Japanese Application No. 2003-413758 filed December 11, 2003, and Japanese Application No. 2004-

096686 filed March 29, 2004, as well as receipt of the certified copies of the priority documents in this national stage application.

**Rejections Under § 112**

Claim 6 is rejected under 35 U.S.C. §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Office Action asserts that the phrase “a 5-cm RWV vessel is used” is indefinite because it is allegedly not clear what dimension of the vessel is 5 cm. The Office Action asserts that claim 6 will be read as “a 5 cm diameter RWV vessel is used” for the interest of prosecution.

Applicants submit that claim 6 pending prior to the present amendment clearly defines what Applicants consider to be their invention. However, solely for the purpose of advancing prosecution of the present application, Applicants have amended claim 6 to recite “a 5 cm diameter RWV vessel is used” as suggested by the Office Action.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the rejection under §112, second paragraph.

**Rejections Under § 102(b)**

Claims 1-5 and 9 are rejected under 35 U.S.C. §102(b) as being anticipated by Goodwin *et al.* (U.S. Patent No. 5,496,722; hereinafter “Goodwin”) and Schwarz *et al.* (U.S. Patent No. 5,026,650; hereinafter “Schwarz”) “with support from Unsworth *et al.*” (Nature Medicine, 4:

901-907, 1998; hereinafter “Unsworth”). The Office Action appears to have cited two references because it is asserted that Goodwin expressly incorporates Schwartz (column 8, lines 5-10 of Schwartz) so that both Goodwin and Schwartz are treated as the same document when applied against the current claims.

The Office Action asserts that Goodwin teaches a method of culturing bone marrow cells into a 3D tissue in a simulated microgravity environment (column 4, lines 36-45 of Goodwin). The Office Action contends that this microgravity environment is simulated by a RWV (column 8, lines 5-10 of Goodwin) and that the RWVs simulate an environment of  $10^{-2}$  of ground gravity as supported by Unsworth at page 902, column 1. Goodwin also allegedly teaches that bone marrow can be obtained from the patient, tissue grown and then transplanted back to the patient (column 5, lines 5-12 of Goodwin).

Applicants note that Goodwin is directed to normal mammalian tissue and culturing processes for developing organ, structural, and blood tissue (abstract of Goodwin). The cells disclosed in Goodwin are grown *in vitro* under microgravity culture conditions and form three dimensional cell aggregates with normal cell function (abstract of Goodwin).

The claimed method is not expressly or inherently disclosed in Goodwin. The process disclosed in Goodwin for tissue engineering is different from the claimed method because the tissue engineering of Goodwin is performed with a culture matrix containing both epithelial cells and mesenchymal cells (column 5, lines 57-63 of Goodwin), and the resulting organ tissue mass also develops cells characterized as angioblasts and epithelial cell brush borders (column 5, line 66 to column 6, line 3 of Goodwin). The resulting tissue obtained from Goodwin is also disclosed to grow anchored to microcarriers joined with cord-like structures of fibroblasts

covered by epithelial cells (column 6, lines 6-8 of Goodwin). In contrast, the claimed method is directed to engineering cartilage tissue by three-dimensionally culturing of bone marrow mesenchymal cells. Also, as disclosed on page 1, lines 15-18 of the present specification, dedifferentiation and conversion into fibroblasts of cartilage cells results in loss of the original phenotype of the cartilage cells.

Additionally, although structural tissue such as cartilage is disclosed to be produced in Goodwin, the structural tissue is prepared from nonhuman embryonic cartilage cells (column 6, lines 22-34 of Goodwin). Likewise, although bone marrow tissue is disclosed to be produced in Goodwin, the bone marrow tissue is prepared from bone marrow cells in which the inoculum of bone marrow includes pluripotential stem cells (column 6, lines 36-40 of Goodwin). In contrast, the claimed method does not use cartilage cells to engineer cartilage tissue, but “involves the use of cells other than cartilage cells” to produce cartilage tissue as disclosed on page 2, lines 21-24 of the original specification, because the use of cartilage cells imposes damage on healthy tissue and is limited by the amount of extraction of cartilage cells. The claimed method allows production of a large quantity of cartilage tissue without damaging healthy tissue by using mesenchymal stem cells from bone marrow instead of autologous cartilage (page 3, lines 2-6 of the present specification).

Furthermore, Goodwin discloses a method for tissue engineering by three-dimensionally culturing cells using RWV, i.e., in a simulated microgravity environment.

However, Goodwin does not demonstrate production of cartilage tissue by culturing bone marrow cells, but production of normal organ tissue, particularly blood tissue, by culturing intestine epithelial and mesenchymal cells (see Column 6 lines 22-34, Column 8 line 63-Column

11 line 22 of Goodwin). Specifically, a three dimensional organ tissue with cord-like structure of fibroblasts covered by epithelial cells is produced by culturing epithelial and mesenchymal cells (see Column 11, lines 20-21 of Goodwin).

Goodwin describes that “[t]he three dimensional tissue mass is developed with functional chondrocytes and stromal cells” using RWV. However, Goodwin only discloses the production of Type IV, IX, X collagens (Column 6 lines 22-34 of Goodwin). Thus, Goodwin does not describe the actual production of cartilage tissue by culturing bone marrow cells.

With regard to the size of the tissue disclosed in Goodwin, the tissue constructed by Goodwin is about 200  $\mu\text{m}$  in diameter (see Fig. 1 of Goodwin) and about 300  $\mu\text{m}$  in diameter (see Fig. 2 of Goodwin). Accordingly, the product disclosed in Goodwin should be called a “spheroid” rather than a “tissue.”

In contrast, the three-dimensional tissue constructed by the method of the present invention has a major axis of over 1 cm which can be used for tissue transplantation. The size of the tissue constructed by the method of the present invention is 30 to 50 times larger than that constructed by the method described in Goodwin. Thus, the method of the present invention is also different from the method described in Goodwin in terms of the size of the resultant tissues.

With regard to the rotation speed described in the method of Goodwin, tissues (spheroids) in the RWV vessel are rotated in accordance with the rotation of the vessel and medium therein. In contrast, the method of the present invention balances the tissues in the RWV vessel in order to maintain their relative positions (see the specification “The rotation speed was frequently adjusted manually by visually inspecting the cell aggregate to maintain a stationary position in a vessel (time course of the rotation speed of an RWV is shown in Fig. 5” at pages 9-10). This

rotation speed enables the production of large tissue with a major axis of over 1 cm in the method of the present invention.

With regard to the Type II collagen versus Type X collagen, Applicants note that among cartilage markers such as Type II, IX, X, and XI collagens, Type II collagen is the most important marker to characterize cartilage. Type X collagen is a marker of hypertrophic cartilage and expressed at a later stage of cartilage differentiation, i.e., in the initiation of bone formation. Therefore, the expression of Type X collagen is not preferable for tissues used for tissue regeneration. Type X collagen is not expressed, but Type II collagen is expressed in the cartilage tissue produced by the method of the present invention. The cartilage tissue shows typical properties of cartilage tissue characterized by, for instance, higher expression of aggrecan, safranin O staining (+), in the present invention.

In contrast, and as discussed above, the three dimensional tissue mass developed with chondrocytes and stromal cells shows no typical cartilage characteristics other than the expression of Type IV, IX, X collagens.

Moreover, mesenchymal cells that may be differentiated into chondrocytes and stromal cells are included in bone marrow cells. The purpose of the present invention is three-dimensional tissue engineering by culturing bone marrow cells in a simulated microgravity environment to control the differentiation of the cells. Thus, the control of differentiation and the cell source are important. As shown in Example 3, rabbit bone marrow cells are cultured for two weeks using RWV and the resultant cartilage tissue is transplanted into an osteochondral defect in a rabbit knee joint. The two-week culture is insufficient for tissue engineering for

transplantation in general. However, the transplanted tissue develops mature cartilage and bone tissue. This is because the differentiation direction of the cells has been controlled (determined).

With regard to Schwarz, Applicants note that Schwarz is directed to a bioreactor system where cell growth microcarrier beads are suspended in zero head space fluid medium by rotation about a horizontal axis (abstract of Schwarz). In this regard, Schwarz is merely incorporated by reference in Goodwin for the general teaching of using a bioreactor to culture tissue. Schwarz does not expressly or inherently disclose the claimed method for engineering cartilage tissue by three-dimensionally culturing of bone marrow mesenchymal cells in a simulated microgravity environment. Therefore, the incorporation of Schwarz for the general use of a bioreactor does not cure any of the deficiencies of Goodwin because Goodwin does not disclose the claimed method.

Similarly, the Unsworth document which is relied upon by the Examiner as support for the RWV environment of  $10^{-2}$  of ground gravity, does not cure the deficiencies in Goodwin and Schwarz. Unsworth is a review article that provides an overview of the use of microgravity for generating macroscopic tissue equivalents for basic and applied medical purposes (page 901, 1<sup>st</sup> paragraph of Unsworth) and disclosure of “ $10^{-2}$  g” is in a general context (page 902, column 1 of Unsworth).

Accordingly, for at least these reasons, Applicants respectfully request reconsideration and withdrawal of the rejection under §102(b).

**Rejections Under § 103**

**1. Goodwin and Schwarz**

Claims 1-5, 8 and 9 are rejected under 35 U.S.C. 103(a) as being unpatentable over Goodwin and Schwarz as applied to claims 1-5 and 9. The Office Action asserts that in addition to the rejection of claims 1-5 and 9 as described above under § 102(b), claim 8 limits the bone marrow cells of claim 1 to be two-dimensionally cultured to confluence, subcultured and then cultured in a simulated microgravity environment. The Office Action asserts that Goodwin teaches that bone marrow cells are cultured in a 2D culture and that one of ordinary skill in the art would recognize that this is a form of sub-culturing cellular bone marrow cells before being added to the RWV and that the “aggregates” of bone marrow referred to in Goodwin are confluent tissues of bone marrow cells. The Office Action asserts that because Goodwin teaches that 2D bone marrow cell cultures have a decreased production over time, it would have been obvious to someone skilled in the art to start the bone marrow culture from the primary cells then transfer it to a 2D culture flask to produce a bone marrow monolayer for aggregates that can be cultured in a RWV. The Office Action contends that Goodwin provides motivation to one of ordinary skill in the art because Goodwin discloses that while it is possible to culture bone marrow monolayers in 2D the bone marrow cell production decreases over time (col 4, lines 20-25). Therefore, it is the Office Action’s position that if Goodwin desires an expansion of the bone marrow cells, it would be obvious to culture the aggregates from a 2D monolayer in a microgravity environment where Goodwin has shown reasonable expectation of success by achieving high cell densities in a 3D structure. Therefore, the invention as a whole would have

been *prima facie* obvious at the time of filing in view of the references listed above and as such claims 1-5, 8 and 9 are not allowable.

Initially, Applicants note that the standard for determining whether a patent claiming a combination of prior art elements would have been obvious focuses on “whether the improvement is more than the predictable use of prior art elements according to their established functions.” KSR International Co. v. Teleflex Inc., 127 S.Ct. 1727 (2007). The reason for combining the elements in the manner claimed may come from sources beyond those contemplated by the applicants, including common sense and common knowledge possessed by one of ordinary skill in the art. Accordingly, a *prima facie* case of obviousness may be rebutted by a showing of unpredicted or unexpected results.

In this case, Applicants note that the claimed method provides an unpredicted result by engineering cartilage tissue from bone marrow mesenchymal tissue that could not be contemplated at the time the invention was made. Goodwin shows that at the time the invention was made, the production of cartilage tissue three-dimensionally in a bioreactor was only contemplated to be derived from cartilage cells (column 6, lines 22-34 of Goodwin). Similarly, the production of bone marrow tissue three-dimensionally in a bioreactor was only derived from bone marrow cells (column 6, lines 36-45 of Goodwin). The three-dimensional production of such tissue in a bioreactor according to Schwarz appeared to address the problem of abnormal cell function known to one of ordinary skill in the art because as disclosed in Goodwin, such three-dimensional production resulted in cell aggregates with normal cell function (abstract of Goodwin).

Furthermore, Goodwin teaches away from the claimed method because as disclosed at column 6, lines 31-34 of Goodwin, structural cartilage tissue is produced from nonhuman

embryonic cartilage cells. In contrast, the claimed method for engineering cartilage tissue “involves the use of cells other than cartilage cells” (page 2, lines 21-24 of the specification), or bone marrow mesenchymal cells. Also, as disclosed in Goodwin at column 3, lines 6-9, “[s]tudies on normal tissue differentiation have indicated an important interaction between epithelial cells and mesenchymal cells and the production and differentiation of cells from fibroblasts.” In this regard, the specification at page 1, lines 15-18 discloses that dedifferentiation and conversion into fibroblasts of cartilage cells would result in loss of the original phenotype of the cartilage cells. Therefore, because the claimed method for engineering cartilage tissue by three-dimensionally culturing bone marrow mesenchymal cells is “more than the unpredictable use of prior art elements”, it would not have been obvious to one of ordinary skill in the art to obtain the claimed invention from either Goodwin or Schwarz.

With regard to claim 8 in which “bone marrow cells are two-dimensionally cultured to confluence, subcultured, and then cultured in a simulated microgravity environment”, Goodwin only describes that cell proliferation decreases with time in two dimensional culture.

Applicants note that Goodwin does not teach or suggest that under a confluent condition, matrix production becomes more active than cell proliferation. The cartilage is generally a tissue containing less cells and rich extracellular matrix (for example, in the case of articular cartilage, water: 65-80%, Type II collagen: 10-20%; proteoglycan: 3-7%, cartilage cell: 1%). Then in order to construct cartilage tissue, plenty of matrix proteins such as Type II collagen are needed, thus, “bone marrow cells are two-dimensionally cultured to confluence, subcultured, and then cultured in a simulated microgravity environment” to construct cartilage tissue. In other words, “subculture up to confluence” is not only for the sufficient cell proliferation but for sufficient production of matrix proteins.

With regard to claim 6, the present inventors have found that the best culture conditions is when “culture is conducted by seeding bone marrow cells at a density of  $10^6$  to  $10^7$  cells/cm<sup>3</sup> at a rotation speed of 8.5 to 25 rpm when a 5-cm RWV vessel is used”. This condition cannot be obtained merely by combining conditions each of which is disclosed in different references without undue experimentation. Applicants also note that TGF- $\beta$  and/or dexamethasone are added as an auxiliary element.

However, even if Goodwin and Schwarz are combined, as discussed above, the combination does not result in the claimed method because neither Goodwin nor Schwarz teach or suggest the unpredicted claimed method for engineering cartilage tissue by three-dimensionally culturing bone marrow mesenchymal cells in a simulated microgravity environment.

Accordingly, for at least these reasons, Applicants respectfully request reconsideration and withdrawal of the rejection under §103(a).

## **2. Goodwin, Schwarz, and Synthecon**

Claims 1-6 and 9 are rejected under 35 U.S.C. 103(a) as being unpatentable over Goodwin and Schwarz as applied to claims 1-5 and 9, and further in view of Synthecon (<http://synthecon.com/products.shtml>, available Feb 5, 2002 as verified by web.archive.org). The Office Action asserts that claim 6 further limits claim 5 by teaching the bone marrow is cultured by seeding the cells at a density of  $10^6$  to  $10^7$  cells/cm<sup>3</sup> at a rotational speed of 8.5 to 25 rpm when a 5 cm diameter RWV vessel is used. Goodwin is asserted to teach that bone marrow cells are added to the RWV at a concentration of  $1\times 10^6$  cells/cm<sup>3</sup> and Schwarz is asserted to teach that the RWV rotates at 10 rpm (Schwarz *et al.*, col 6, lines 35-40). The Office Action asserts

that even though neither Goodwin nor Schwarz teach that the RWV has a 5 cm diameter vessel this would be obvious to one of ordinary skill in the art at the time the invention was made in view of Synthecon which is asserted to teach that RWV culture systems can be made to almost any size required by the experimenter without affecting the physics of the system (Synthecon, page 2) absent any teaching by Applicant concerning the criticality of the dimensions of the RWV listed in claim 6. The Office Action asserts that it would be *prima facie* obvious that one of ordinary skill in the art would recognize that the size of the RWV vessel in claim 6 is a result effective variable that depends on the size of the culture required by the experimenter, which is a matter of routine optimization.

The deficiencies of Goodwin and Schwarz are discussed above. With regard to Synthecon, Applicants note that the web page merely discloses that culture medium continuously flows through the rotating cell culture vessel and that medium can be exchanged, sampled, or modified (page 1, 1<sup>st</sup> paragraph of Synthecon). The Synthecon document appears to disclose that different applications have specific needs and modifications on a case by case basis may be requested. There is nothing in Synthecon that teaches a RWV culture system, instead, Synthecon teaches a RollerCell 40 which is “a self-contained, automated roller bottle processing system” that is known in the art to be used for large scale monolayer culturing of cells, and not the RWV which is a bioreactor that rotates along the horizontal axis of the cylinder to provide three-dimensional culture of cells. (See Melero-Martin, J.M. and M. Al-Rubeai, *In vitro* Expansion of

Chondrocytes, Topics in Tissue Engineering, Vol. 3, Chapter 2, page 22, 2007).<sup>1</sup> Therefore, the addition of the Synthecon document only further teaches away from the claimed method.

Accordingly, for at least these reasons, Applicants respectfully request reconsideration and withdrawal of the rejection under §103(a).

### **3. Goodwin, Schwarz further in view of Yan and Simpson**

Claims 1-5, 7 and 9 are rejected under 35 U.S.C. 103(a) as being unpatentable over Goodwin and Schwarz as applied to claims 1-5 and 9 above, and further in view of U. S Patent Application Publication No. 2002/0168763 to Yan (hereinafter “Yan”) and U. S. Patent Application Publication No. 2002/0090725 to Simpson *et al.* (hereinafter “Simpson”). The Office Action asserts that the description and rejection of claims 1-5 and 9 are described in the 35 U.S.C. § 102(b) rejection above. The Office Action appears to assert that because claim 7 further limits the method of claim 1 by requiring TGF-  $\beta$  and/or dexamethasone in the culture medium, it would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the invention of Goodwin in view of Simpson and Yan.

With regard to Simpson, the Office Action asserts that although Goodwin does not specifically teach TGF-  $\beta$ , Simpson teaches the addition of TGF-  $\beta$  to the culture medium (paragraph 98 of Simpson) to grow collagen matrices in a microgravity reactor (paragraph 207 of Simpson) that contain cells from bone marrow (paragraph 204 of Simpson). The Office Action contends that the motivation comes from the teaching in Goodwin that “various growth factors”

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<sup>1</sup> In accordance with M.P.E.P. §609(c), the document cited herein in support of Applicants’ remarks is being submitted as evidence directed to an issue raised in the Office Action, and no fee pursuant to 37 C.F.R. §§ 1.97 and 1.98, or citation in a FORM PTO-1449 or SB/08 is believed to be necessary.

may be added to the culture medium to “emulate *in situ* conditions” (column 4, lines 3-5 of Goodwin). In addition, the Office Action asserts that a reasonable expectation of success is provided by Simpson because Simpson teaches addition of TGF-  $\beta$  to the culture and one of ordinary skill in the art would recognize that TGF-  $\beta$  would be present in the body where bone marrow cells are cultured.

With regard to Yan, the Office Action appears to assert that although Goodwin does not teach the addition of dexamethasone to culture media, it would have been obvious for one of skill in the art to add dexamethasone because Yan teaches the addition of dexamethasone to culture media (Yan, paragraphs, 178 and 330) that grows bone marrow cells (Yan, paragraph 85) in a microgravity environment (Yan, paragraph 111) for bone marrow transplantation (Yan, paragraph 43) which is the same purpose as Goodwin.

In response, and as discussed above, the further combination of Goodwin and Schwarz with Simpson and Yan does not cure the deficiencies of the Goodwin and Schwarz documents.

Applicants note that Simpson is directed to formation and use of electroprocessed collagen as an extracellular matrix in forming engineered tissue and organs (abstract of Simpson). Although Simpson discloses that TGF-  $\beta$  may be added to a collagen matrix having electroprocessed collagen, TGF-  $\beta$  is disclosed amongst a general list of other growth factors (paragraph [0098] of Simpson). Furthermore, bone marrow stem cells are disclosed by Simpson to be combined in an electroprocessed collagen material to “make organs or organ-like tissue such as livers or kidneys (paragraph [0204] of Simpson), and the use of “simulated microgravity” in a bioreactor is disclosed by Simpson in regard to vascular prosthesis (paragraph [0207] of Simpson). There is nothing in Simpson that would provide one of ordinary skill in the

art a reason to modify the method of Goodwin to obtain the unpredicted claimed method for engineering cartilage by three-dimensionally culturing bone marrow mesenchymal cells.

Applicants note that Yan is directed to pluripotent homozygous stem (HS) cells and method and materials for making the same (abstract of Yan). Yan discloses that HS cells may be induced to differentiate *in vitro* or *in vivo* into various types of tissues (paragraph [0085 of Yan]), but there is nothing in Yan that teaches the claimed method. Also, it appears the Office Action is mistaken regarding the addition of dexamethasone to culture media because paragraphs [0177] and [0178] of Yan discloses that retinoic acid (RA) is a prerequisite for commitment of HS cells into the adipocyte lineage, so that RA cannot be substituted by hormones or compounds known to be important for terminal differentiation, and addition of dexamethasone “leads to a low level of adipogenesis (5%)” (paragraph [0178] of Yan). For this reason, Applicants note that Yan teaches away from using dexamethasone in culture. In addition, Applicants note that adipogenesis is known in the art to be the development of fat cells from preadipocytes, which is not related to the claimed method of engineering cartilage tissue by three-dimensionally culturing bone marrow mesenchymal cells. Therefore, there is no teaching or suggestion in Yan that would provide one of ordinary skill in the art with a reason to modify the method of Goodwin, Schwarz, and Simpson with the method of Yan to obtain the unpredicted claimed method for engineering cartilage by three-dimensionally culturing bone marrow mesenchymal cells.

However, even if Simpson and Yan are combined with Goodwin and Schwarz, the combination would not result in the claimed method. At most, one of ordinary skill in the art would obtain cells grown *in vitro* under microgravity culture conditions with a bioreactor to form

three dimensional cell aggregates in which the culture matrix is without dexamethasone containing both epithelial cells and mesenchymal cells in addition to electroprocessed collagen, and the resulting organ tissue mass contains cells characterized as angioblasts and epithelial cell brush borders.

Accordingly, for at least these reasons, Applicants respectfully request reconsideration and withdrawal of the rejection under §103(a).

**CONCLUSION**

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

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# *In Vitro Expansion of Chondrocytes*

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## *Summary*

**A**n alternative therapy for the repair of damaged articular cartilage resides in the tissue engineering approach. However, routine tissue culture methodologies can hardly cope with the scale of cell production required for the manufacture of engineered cartilage tissue products. Consequently, *in vitro* cell expansion has become an essential step in the process of tissue engineering of articular cartilage and the optimization of expansion protocols is a fundamental issue that needs to be addressed. In particular, both the finding of alternative sources of stem/progenitor cells and the development of feasible large scale cell expansion processes are mandatory requirements for a sufficient production of chondrocytes. In this review we delineate the progress that has been made to date and the challenges remaining for the successful production of the large number of articular chondrocytes that would be eventually required by the cartilage tissue engineering industry.

**Keywords:** tissue engineering; chondrocytes; chondroprogenitors; expansion; stem cells



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## Introduction

### Articular cartilage

Articular cartilage, also called hyaline cartilage, is the thin, smooth, glistening white tissue that covers the surface of all the diarthrodial joints in the human body. It has an incredibly low coefficient of friction which, coupled with its ability to bear very large compressive loads, makes it ideally suited for placement in joints, such as the knee and hip. The smoothness and thickness of the cartilage determines the load-bearing characteristics and mobility of the joints. It is a tissue only a few millimeters thick, but with excellent wear characteristics. Its mechanical and structural capacity depends on the integrity of its extracellular matrix. Extracellular components of collagen, proteoglycans, non-collagenous proteins and water provide the shear, compressive, and permeability characteristics of the articular cartilage (1-3). Chondrocytes sparsely distributed throughout the matrix of structural macromolecules work together with hydrated extracellular glycosaminoglycans to attract and then sequentially extrude water. This charged mechanical interaction permits cartilage to perform its mechanical functions without appreciable wear (1-3). The functions of articular cartilage include load transmission and distribution, smooth articulation, and aid in lubrication (1, 4). Load transmission and distribution is due to the ability of the structural matrix to deform, which leads to increased joint contact areas and distributed mechanical stresses (1). It also has the ability to respond to applied loads through fluid exudation and redistribution within the interstitial tissue.

Articular cartilage is not a homogeneous tissue. Instead, it has a very complex composition and architecture that permits it to achieve and maintain proper biomechanical function over the majority of a human lifespan. Water constitutes between 65-80% of the entire wet weight of articular cartilage (5) and is about 15% more concentrated at the surface than in the deeper zones (1). Chondrocytes are the only cells of articular cartilage, and they are responsible for the production of the extracellular matrix. Distributed throughout the matrix, chondrocytes compose less than 5% wet weight (1, 2). Collagen makes up about 15-22% of the wet weight and contains 90-95% type II collagen fibers with a small percentage of types IX and XI (1, 2). This is what provides the high tensile stiffness, strength and resiliency of the tissue. Proteoglycans constitute about 4-10% of the total wet weight and are a mix of large aggregating (50-85%) and large non-aggregating (10-40%) proteoglycans (1, 2). They are responsible for pressure elasticity and charged interactions with water (3, 6). Non-

collagenous proteins, elastins, integrins and other macromolecules of protein are responsible for the matrix organization and maintenance (2). The distribution and arrangement of these components, however, is not uniform. Instead, articular cartilage is divided into four zones: superficial, middle, deep, and calcified. The superficial zone is characterised by flattened chondrocytes, relatively low quantities of proteoglycans, and high quantities of collagen fibrils arranged parallel to the articular surface (7). The middle zone, in contrast, has round chondrocytes, the highest level of proteoglycans among the four zones, and a random arrangement of collagen (8). The deep zone is characterised by collagen fibrils that are perpendicular to the underlying bone, and columns of chondrocytes arrayed along the axis of fibril orientation (9). The calcified zone is partly mineralised, and acts as the transition between cartilage and the underlying subchondral bone (9). It is the composition and highly complicated interaction of these components that makes regeneration and replacement techniques of articular cartilages challenging.

### *Diseases and injuries of articular cartilage*

Diseases of the joints affect over 500 million people worldwide (10). Articular cartilage lesions are generated during the course of many joint diseases, notably osteoarthritis, in conjunction with a large number of genetic or metabolic conditions, such as acromegaly, Paget's Disease, the Stickler-Syndrome and hemophilia (11), or as a result of trauma. Traumatic lesions may occur directly or indirectly in consequence of an intraarticular fracture, a high-intensity impact or following ligament injuries (11, 12). Some of the most common diseases related to articular cartilage lesions are arthritis, degenerative joint disease or osteoarthritis, rheumatoid arthritis and osteochondritis dissecans. Approximately 46 million people in the U.S. suffer from some form of arthritis, and that number is expected to rise to 60 million by the year 2020, which will constitute nearly 20% of the projected population (13). This disease causes joint pain, stiffness, and depending on its severity, it can turn simple, everyday activities into arduous tasks. Degenerative joint disease (DJD) represents over 40% of the arthritic patient population (13). It affects nearly 33 million people in the U.S. and accounts for an estimated 7.3 million physician visits per year (13). In simplest terms, the disease is born of cyclic wear-and-tear that results in the breakdown of cartilage at the ends of bones that in turn cause pain in the joint. The disease is commonly referred to as osteoarthritis (OA). The systemic condition, or rheumatoid arthritis, is a far less common autoimmune condition. Rheumatoid arthritis is not an inherited disease, although researchers believe that some people have genes that make them susceptible to the disease

(13). People with these genes will not automatically develop rheumatoid arthritis. Rheumatoid arthritis (RA) affects an estimated one to two percent of the world's population (10). Osteochondritis dissecans (OCD) is a disorder in which a fragment of cartilage and subchondral bone separates from an articular surface (11). The etiology is uncertain, although trauma and ischemia have been implicated. It has been estimated that 4% of all cases of osteoarthritis of the knee diagnosed in men were the direct result of OCD (10).

Articular cartilage injuries may occur as a result of either traumatic mechanical destruction or progressive mechanical degeneration. With mechanical destruction, a direct blow or other trauma may injure the articular cartilage. Occasionally, an articular cartilage fragment breaks loose from the underlying bone. This fragment, called a loose body, may float in the joint, interfering with normal joint motion. Progressive mechanical degeneration of the articular cartilage, commonly referred to as wear and tear, occurs with the progressive loss of the normal cartilage structure and function. This initial loss begins with cartilage softening and proceeds to actual fragmentation of the cartilage. A loss of articular knee cartilage continues, the underlying bone has no protection from the wear and tear of daily living and begins to break down, an event that may lead to OA. Causes of progressive mechanical degeneration of the articular knee cartilage include high-impact twisting injuries, joint instability and inadequate muscle strength or endurance (13).

### *Self repair capability of articular cartilage*

In articular cartilage, the combination of the lack of blood supply and a few cells distributed widely amongst a dense extracellular matrix leads to a limited ability to heal (1, 11, 14, 15). The usual inflammatory response of hemorrhage, formation of fibrin clot, cellular production and migration of mesenchymal cells is absent (11). However, spontaneous repair of articular cartilage may eventually occur depending on the depth of the lesion. While surface defects that do not penetrate the subchondral bone have to rely on sparsely populated chondrocytes for matrix remodeling, deeper lesions may introduce a blood supply from the well-vascularised subchondral bone (16). With the blood come various types of stem cells and fibrocytes that modulate to fibrochondrocytes (17-19). In addition, large quantities of growth factors are also released from the bone (20-22), playing an important role in initiating the repair response. Nevertheless, these combination of cells and growth factors produce a relatively disorganized network of collagen fibres partially filling the defect with structurally

weak tissue (16). The heterogeneous composition and inferior biomechanical properties of spontaneously formed cartilage undoubtedly contribute to its functional incompetence and perishability (16). Apart from the depth and degree of damage, other factors such as age, traumatic or chronic condition, associated instability, and genetic predisposition are also factors affecting healing of cartilage (1, 2, 11). For example, age affects healing in part because in newborns, the multi-functioning mesenchymal stem cells needed for healing account for 1 in every 10,000 cells in bone marrow and reduces to 1 in 100,000 in teens, 1 in 400,000 by age 50 and 1 in 2 million in an 80 year old (16). Clearly, for a successful (reproducible and durable) repair result, a more homogeneous repair cell population, which is capable of producing hyaline-like cartilage, is required.

**Table 1. Current therapeutic techniques for the treatment of articular cartilage defects**

Methods	Techniques	Disadvantage
<b>I-Lavage and arthroscopy</b>		
Lavage	Irrigation of a joint with solutions of NaCl, Ringer or Ringer and lactate.	Only short term pain relief. Not solid evidences of any biological or repair activity being instigated.
<b>II-Debridement</b>		
Shaving and debridement of cartilage	Mechanical removal of diseased chondral tissue.	Instability of the joint. High rate of re-injury.
Thermal chondroplasty (laser and radiofrequency)	Alternative debridement by heat or radiofrequency energy.	Joint tissue necrosis by thermal damage.
<b>II-Marrow Stimulation Techniques</b>		
Abrasion chondroplasty	All these techniques involve surgical access to the subchondral bone until bleeding occurs inducing spontaneous repair responses.	Repair tissue becomes fibrocartilaginous (less durability and strength). Restriction of mobility.
Multiple drilling (Pridie drilling)		
Microfracture		
<b>III-Transplantation</b>		
Autologous grafting (periosteal/perichondrial/osteochondral plug transfer)	Replace lost cartilage with tissue grafts from own patient.	Poor fixation of graft to damage area. Inflammatory and joint mobility restriction. Donor site morbidity and availability.
Allogeneic grafting	Replace lost cartilage with tissue grafts from different individuals.	Immunological reaction and disease transmission. Limited supply of graft. Handling and storage of frozen tissue.
<b>IV-Cell-based therapies</b>		
Autologous Chondrocyte Transplantation	Removal of patient own cells by biopsy. <i>in vitro</i> cell culture and implantation into defect under a periosteal patch.	Expensive treatment. Two surgical procedures. Potential damage to surrounding cartilage. Restricted activity to allow cell integration. Long term degeneration.
Marrow Stromal Cells		Only animal models so far.

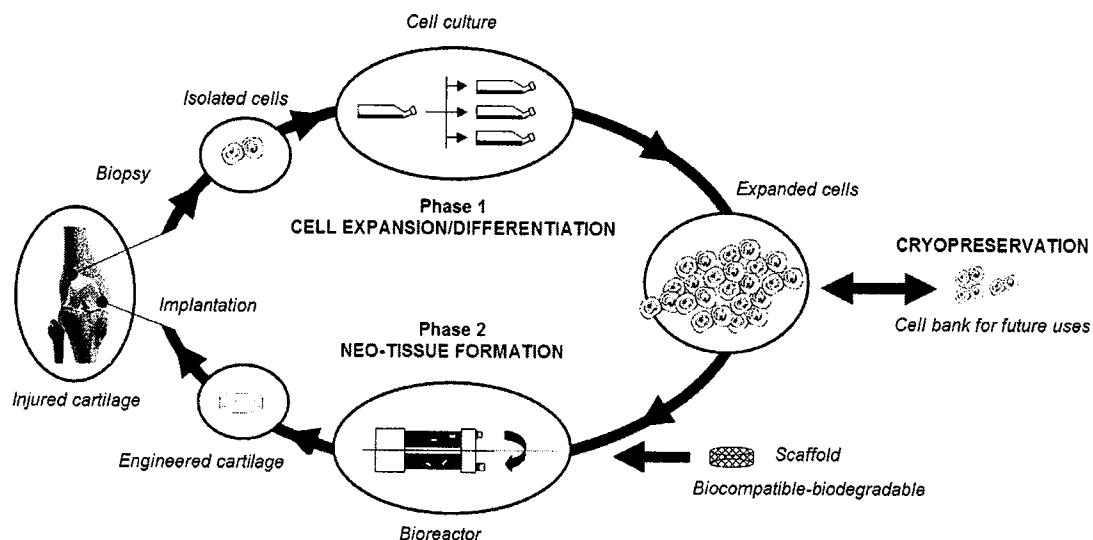
### ***Currently available repair techniques***

Articular cartilage injuries affect more than 1 million people each year in the U.S., with approximately 600,000 surgical procedures being performed annually for the treatment of these injuries (10). The limited ability of articular cartilage to self repair has led to a wide variety of treatment approaches for focal chondral defects with varying levels of success (16, 23). These techniques may be classified either as 1) debridement of loose or impinging chondral flaps; 2) stimulation of the intrinsic repair mechanism from subchondral bone; 3) transplants to fill the defect with autografts or allografts; 4) cell-based therapy to regenerate the chondrocytes and surrounding matrix; or 5) combinations of these techniques with growth factors or biologically active carriers to influence the repair process. Most of these techniques are briefly described in Table 1.

### ***Tissue engineering approach***

An alternative therapy for the repair of damaged articular cartilage resides in the tissue engineering approach. Tissue engineering is an interdisciplinary field that applies principles and methods of engineering and the life sciences toward the development of biological substitutes that restore, maintain and improve the function of damaged tissues and organs (24). Such tissue reconstitution process can be conducted either entirely *in vitro* or partially *in vitro* and then completed *in vivo*. In Figure 1 a schematic representation of this technology is depicted. In the process of articular cartilage tissue engineering, two main phases can be identified. A first phase where few isolated cells need to be expanded in order to provide sufficient cells and a second phase where a cartilage is engineered either inside the body (*in vivo*) or in a cell culture (*in vitro*) using an appropriate scaffold. Tissue engineering can potentially use cells taken either from the patient (autologous) or from a donor (allogeneic), and these cells may be mature cells (e.g., chondrocytes) or immature cells (e.g., mesenchymal stem cells). Scaffolding technologies plays a crucial role in this technology. Biocompatible scaffolds, the most common of which involve collagen or polymer (25), allow cells to be grown in systems such as rotating bioreactors (26, 27), and spinner flasks (28). Expanded cells are seeded onto three dimensional scaffolds to form cell-polymer constructs, which are cultured *in vitro* and then used either as implants or for *in vitro* research. All these systems are designed not only to maintain the proper culture medium and environmental conditions

for cell propagation and growth, but also to transport the living construct to the ultimate operating site.



**Fig. 1.** Schematic representation of articular cartilage tissue engineering. Cells are harvested from the articular cartilage, expanded in tissue culture flasks and seeded on a biocompatible-biodegradable scaffold. The cell/scaffold constructs undergo a period of dynamic tissue culture in a bioreactor prior to implantation. This figure was adapted from reference (55) with permission.

Since 1991, when Vacanti *et al.* (1991) produced new hyaline cartilage from bovine chondrocytes on a polymer scaffold, tissue engineering of articular cartilage has notoriously evolved. A wide variety of scaffolds have been used to produce cartilage *in vitro*. Form and composition of scaffolds range from non-woven meshes and foams of alpha-hydroxypolymers (29, 30), polyglactin (31) or hyaluronan alkyl esters (32) to photocrosslinked hydrogels (33) and sponges based on different types of collagen and glycosaminoglycans (34). Scaffolds filled with cells embedded in a fibrin or alginate gel have also been proposed (31, 35). The design of specific bioreactors have also improved the formation of cartilage tissues by providing an efficient and spatially uniform cell seeding and by allowing a better diffusion of nutrients to the cells into the scaffold. Cartilaginous tissue has been repeatedly engineered *in vitro* by using articular chondrocytes cultured on three-dimensional biodegradable scaffolds in tissue culture bioreactors. The success of the development of structurally suitable cartilaginous matrix *in vitro* has been found to depend on many culture conditions including cell seeding density and conditions (28, 36-38), the *in vitro* culture environment (28, 39, 40), bioreactor design (41) and the mechanical deformation and stimulation of engineered tissues under controlled conditions (42-47). The advances in all

**Table 2. Selected current and emerging chondrocyte transplantation products. (Source: Medtech Insight, 2003)**

Company	Product (s)	Description	Regulatory Status
Smith & Nephew	NeoCyte	Developed in collaboration with Advance Tissue Sciences, this product is designed to circumvent the need for total knee replacement	Preclinical trials. In 2003 both companies filed for IDE approval
	Stromal Tissue Culture System	Three-dimensional stromal tissue culture system	Development stage
Cell Transplantation Innovations/ProChon Biotech	BioCart	Designed for the propagation of human cartilage cells as a basis for autologous chondrocyte transplantation to treat articular damage in knees	Approved for commercial use in Israel
Co.don Tissue Engineering	Co.don chondrotransplant	Cultured autologous cartilage cell transplant for repairing focal cartilage defects in the knee	Clinical trials
Genzyme	Carticel	Autologous cultured chondrocytes	FDA approved in 1995
	Carticel II	Second-generation product	Phase II clinical trials
	Quick Tack	Tack for attaching periosteum. Osteoarthritis treatment	Phase II clinical trials
IsoTis	VivesCart	Second-generation tissue engineering cartilage developed in conjunction with Massachusetts Institute of Technology	Preclinical trials. FDA approval expected by 2005
Isto Technology	Istocyte Chondral Allograft	Three-dimensional articular cartilage graft formed using pediatric cadaveric donor chondrocytes	Preclinical trials
Verigen Transplantation Service International	CACI	One-step procedure that replaces excised damaged tissue with a purified resorbable porcine collagen membrane	CE-Mark approved
	MACI	Two-steps process that involves culturing autologous chondrocytes and inserting them into a collagen matrix which is then implanted into the defect site	Clinical trials
	MACI (A)	Alternative approach to the MACI procedure	Development stage

FDA = Food and Drug Administration. IDE = Investigational Device Exemption. CE = *Conformité Européene*. CACI = Collagen-covered Autologous Chondrocyte Implantation. MACI = Matrix-activated Autologous Chondrocyte Implantation. Advance Tissue Science' assets were acquired by Smith & Nephew in May 2003.

these areas have allowed the achievement of suitable (biochemical and histological) engineered constructs. Such engineered constructs showed both high fractions and homogeneous distributions of proteoglycan and collagen, with collagen type II representing the dominant fraction of total collagen (39).

Widespread discourse about the early experiments with tissue engineering has generated public demand and expectations that engineered tissues will be available before long. There are, however, critical hurdles that still need to be overcome. In the case of engineered cartilage, it would seem preferable to avoid harvesting of normal tissue and have a single operation for the implantation of the engineered tissue. In addition, consideration of the limited proliferative and regenerative capacity of adult chondrocytes and their potential dedifferentiation upon expansion leads to the goal of an alternate source of cells. Regarding the scaffolds, only limited materials are currently available and approved by the regulatory authorities (13), and innovative synthetic materials, such as polypeptides or novel biodegradable polymers, need to be further improved with regard to: i) nature of polymers favoring cell-substrate interactions and bioactive molecules release; ii) pore size and scaffold geometry (foam, mesh or gel) that affects the induction/maintenance of the chondrocyte phenotype (48).

Finally, there remain difficulties in the incorporation of neo-cartilage with adjacent healthy tissue (49). There is incomplete understanding of the relationship between cartilage and vascular response to wounding. Engineered cartilage needs to attach to the implantation site without evoking an angiogenic response. Enhancement of cartilage antiangiogenic activity may be needed, but these metabolic processes are not yet fully understood (49).

At the moment, cell-based therapies involving the implantation of cells are the centre of most of the innovations emerging in the field of articular cartilage repair (Table 2). However, some three-dimensional tissue engineering products can be currently found in their development stages (e.g., Stromal Tissue Culture System, Smith&Nephew; Table 2). Success in tissue engineering technology would obviate the need for tissue transplantation, and if the appropriate precursor cell pools could be obtained from embryonic, fetal or adult allogeneic sources, then the numerous problems associated with the use of donor tissue would be avoided.

### ***Challenge of cell expansion in cartilage tissue engineering***

One of the challenges that tissue engineers will have to address in the near future is the development of feasible large scale cell expansion processes. The estimated number of articular cartilage incidences worldwide is around 30 million cases of knee osteoarthritis and 1.2 million cases of focal defects every year (50). If tissue engineering products are to be used in the future for the treatment of all these incidences, it is crucial to estimate the scale of cell production needed for all these repair procedures, essential question that is rarely approached in reported tissue engineering studies. Such estimation has been presented in Table 3. As a guideline for this estimation, it has been presumed a typical size of focal defect in articular cartilage of 4 mm thick and 40 mm in diameter, while for osteoarthritis defects, two implants (opposing joint surfaces) of 4 mm x 40 mm would be required. In the literature, few systematic studies have addressed the effects of the initial cell density on cartilage formation. It is known that polylactide-polyglycolide scaffolds seeded with a density of less than 10 million cells per ml will typically result in little, if any cartilaginous material (36, 51).

**Table 3. Estimation of cell necessities for tissue engineering treatments**

Indication	Incidence of surgical procedures Worldwide	
Focal defects in Articular Cartilage	$1.2 \times 10^6$	
Osteoarthritis (OA of the knee)	$3 \times 10^7$	
Tissue engineering treatment	Number of implants	Cells required <sup>a</sup>
<b>Focal defects in Articular Cartilage</b>		
Typical human defect size (4mm x 40mm) <sup>b</sup>	1	$3.2 \times 10^8$
10% of focal defect incidences <sup>c</sup>	$1.2 \times 10^5$	$3.9 \times 10^{13}$
<b>Osteoarthritis</b>		
Typical human OA (2 x 4mm x 40mm) <sup>b</sup>	2	$6.4 \times 10^8$
10% of OA defect incidences <sup>c</sup>	$3 \times 10^6$	$1.9 \times 10^{15}$

<sup>a</sup> 64 million cells/cm<sup>3</sup>

<sup>b</sup> Typical sizes provided by Smith&Nephew Research Centre, York, UK

<sup>c</sup> Assuming 10% of incidences are appropriate for treatment

As the cell seeding density increases, the integrity of the cartilaginous product appears enhanced and distribution of the cartilaginous material more homogeneous. Of course, not all of the cells in initial seeding solutions attach to scaffolds. Whether the attached cells represent a subpopulation that exhibits some preferential property has not been addressed. However, as a means to get to an end product, seeding at high cell density seems to be

desirable. One study that systematically tested for the effect of cell seeding density on cartilage formation revealed that seeding scaffolds at a density ranging from 20 to 100 million cells per ml resulted in formation of clinically appropriate cartilage when implanted subcutaneously into nude mice; lower seeding density (e.g., 10 million cells per ml) exhibited reduced cartilaginous formation when assessed by wet weight and thickness (36). Consequently, in the estimations presented in Table 3, a seeding density of 64 million cells/ml has been considered as appropriate. This value of seeding density would result in cell demands of 320 and 640 million cells for the treatment of one single focal defect and osteoarthritis defect respectively. Although not all the registered incidences may be suitable for tissue engineering treatments, a recent study have shown that approximately 10% of all knee arthroscopies showed cartilage defects that may be appropriate for cartilage repair procedures (52). Therefore, considering just 10% of all the surgical procedure incidences, the total demand of cells that the production of tissue engineering products would have to face are  $3.84 \times 10^{13}$  and  $1.92 \times 10^{15}$  cells for focal defects and osteoarthritis defects respectively (Table 3). The question is: can routine tissue culturing methodologies cope with this scale of cell production? If we think about autologous applications and assume the possibility of harvesting 2 million articular chondrocytes from a donor's biopsy (53), single treatments of our presumed cartilage defects would require a 160 fold (focal defect) and 320 fold (osteoarthritis) multiplication of the donor cells, values that seems to be achievable with standard culture methodologies (53). However, the future of cartilage tissue engineering may not be in the use of adult autologous chondrocytes applications. The limitations of adult chondrocytes to maintain their phenotype expression and differentiation ability after extensive expansion *in vitro* has led to the investigation of the potential use of pluripotent stem cells and progenitor cells as a source for tissue engineering. In addition, autologous applications include the need for two surgical procedures (biopsy followed by implantation), and it has associated an expensive cost (16). Although success in tissue engineering would eventually obviate the need for tissue transplantation, only if the appropriate precursor cell pools are obtained from embryonic, fetal or adult allogeneic sources, then the numerous problems associated with the use of donor tissue would be avoided. It would be especially in these potential allogeneic applications where the expansion of the cell population *in vitro* becomes an essential step in the development of feasible large scale process of tissue engineering of articular cartilage. Both optimization of the culture conditions and the validation of a cryopreservation procedure to form cell banks are fundamental challenges that need to be addressed (54, 55).

## ***Dedifferentiation and redifferentiation of chondrocytes***

### ***Dedifferentiation***

In the process of cartilage tissue engineering, it is important to ensure that the expanded cell population retains its phenotypic function. However, a major problem of mammalian articular chondrocytes in culture is the phenomenon of dedifferentiation (56). As a result of this process of dedifferentiation, chondrocytes isolated from their tissue-specific extracellular matrix fail to produce cartilage matrix after extensive expansion in monolayer culture. After suspension in growth medium chondrocytes adhere at the bottom of cell culture plastic wares (monolayer cultures) where they can grow and proliferate to form a confluent cell layer. However, the cells lose their morphology as well as their biochemical and functional properties. Such dedifferentiated cells behave completely differently compared to the cells in their original tissue environment. After a few days in monolayer culture, they begin to change their appearance to fibroblast-like morphology (57). The typical formation of chondrons and pericellular matrix is not observed in monolayer culture. Biochemical investigations reveal a switch of collagen synthesis. Thus, instead of the cartilage-like collagen type II, cultured chondrocytes mainly synthesize collagen type I and III which are absent from normal cartilage (58). Also the dedifferentiated chondrocytes present gradual shift from the synthesis of large aggregating proteoglycans (aggrecan) to low molecular weight proteoglycans (versican) and a decrease of alkaline phosphatase (AP) activity (57, 59, 60). This phenomenon has been particularly studied in culture. Dedifferentiation occurs when chondrocytes released from their extracellular matrix are cultured under conditions that promote a flattened cell morphology, such as at low cell density in monolayer, and it is gradually manifested as soon as at passage 1 (61). Although the mechanisms involved the dedifferentiation process are not fully understood, it is believed to be mediated by the formation of actin stress fibers which occurs when the cells spread on an adhesion-permitting substrate (59, 62).

This phenomenon of cell dedifferentiation is probably the most important limitation on the expansion potential of adult articular chondrocytes in tissue engineering applications, where extensive expansion would be eventually necessary for the development of feasible large scale operations. Moderate seeding densities and frequent subcultivations usually optimize growth of most cell types. However, if that approach is used for chondrocytes, there is a

progressive, and often irreversible loss of function (49, 60). In addition, chondrocytes derived from articular cartilage biopsies have only a limited proliferative potential. Not only they dedifferentiate upon repeated passaging (60), but also the number of cell divisions chondrocytes undergo *in vitro* decreases with age (63). It has been known for many decades that chondrogenesis can be enhanced if chondrocytes are seeded at high density (64), suspended in solution (65), isolated chondrons (66), or culture as pellets (56). However, conditions that favor maintenance of phenotype are usually not those that favor increases in numbers (67). As a result, there may be limitations in the numbers of suitable cells that can be grown *in vitro* for subsequent repair of cartilage defects.

### ***Redifferentiation***

Dedifferentiated chondrocytes appear to be very similar to fibroblasts (68). However, in contrast to fibroblasts they can still redifferentiate to normal chondrocytes. Expanded chondrocytes in monolayer culture are probably still determined by a cell memory system to form cartilage in a favorable environment. Although the mechanisms involved in restoration of the differentiated phenotype have not been fully elucidated yet, the dedifferentiation process is believed to be mediated by the formation of actin stress fibers which occurs when the cells spread on an adhesion-permitting substrate (59, 62). It is also well documented that dedifferentiation can be prevented or reversed (redifferentiation) by culturing chondrocytes under conditions that inhibit cell flattening, preventing the formation of stress fibers (69-73). Some of these culture conditions are:

### ***High cell density cultures***

The beneficial effect of cell density on the phenotype stability of articular chondrocytes was early investigated in monolayer cultures (61). Briefly, after three weeks in culture, chondrocytes grown at high density expressed predominantly large proteoglycans that aggregated with hyaluronic acid, whereas in low-density cultures a smaller, non-aggregating form was present. Additionally, both high and low density were expressing type I collagen, although the high-density cells also had an extensive extracellular matrix of type II collagen. These early observations supported the conclusion that high seeding density stabilises the chondrocyte phenotype to a greater extent than low seeding density. They also suggested that

enhanced dedifferentiation at low density may be due to cell spreading, rather than to selective proliferation of a phenotypically unstable subpopulation of cells.

#### *Pellet cultures*

Articular chondrocytes have also been maintained in high-density aggregated cultures (74-77). This system has been reported to support cell proliferation while maintaining the chondrocyte phenotype (78, 79), although the strong interactions cell-cell generated during this kind of culture make the harvesting of viable chondrocytes difficult to achieve.

#### *Hydrogel cultures*

Dedifferentiated chondrocytes can reexpress phenotypic markers of the articular chondrocyte when they are cultured in three-dimensional hydrogels such as agarose, collagen and alginate (80). Dedifferentiated chondrocytes propagated as a suspension culture in agarose gels re-expressed a differentiated phenotype where both collagen and proteoglycan returned to rates exhibited by primary chondrocytes (60). According to these authors, approximately 80% of the cells survived the transition from the flattened morphology of anchorage-dependent culture to the spherical morphology of anchorage-independent culture and then deposited characteristic proteoglycan matrix domains. The rates of proteoglycan and collagen synthesis returned to those of primary chondrocytes, demonstrating a complete return to the differentiated phenotype. Type I collagen, presumably because of its availability in large quantities relative to other collagen types, has also been utilized as a gel substance for culture of chondrocytes. Collagen gels have been demonstrated to support chondrocyte proliferation (81-84), although data addressing the ability of collagen gels to support the synthesis of cartilaginous material *in vitro* is minimal. Matsusaki *et al.* (1998) reported that even under the use of basic fibroblast growth factor (bFGF) for stimulation of proliferation, chondrocytes in collagen gel culture were able to maintain the differentiated phenotype for 4 weeks, although other authors reported that depending on the type of collagen gel used, the percentage of cells that maintained a chondrocyte-like morphology after 1 week in culture varied from 40 to 90% (81). Therefore, the effect of collagen gels on chondrocyte phenotype and the capacity of redifferentiation remain an issue. Finally, it has been also reported that articular chondrocytes remain metabolically active when cultured in alginate beads (85-87). Although there seems to be an initial cell loss after transfer to the alginate beads, the remained cells are able to proliferate and maintain their typical chondrocyte phenotype (88). The potential of this system to redifferentiate the chondrocytes has also been investigated by cultivating them in

alginate beads after monolayer expansion. This resulted in the reexpression of two main markers of differentiated chondrocytes (aggrecan and type II collagen) although several weeks were necessary for total suppression of type I and III collagen synthesis (indicators of a modulated phenotype) and to reach a steady state of cell proliferation and synthesis of fully differentiated proteoglycans (89, 90). Additionally, the extent of proliferation and redifferentiation were seemed to be dependent on the formation of clonal populations of chondrocytes and correlated inversely with the initial cell seeding density (91).

### *Three-dimensional cell carriers*

As with chondrocytes cultures in hydrogels, culturing chondrocytes on appropriated carriers that allow a three-dimensional distribution of the cell and hence inhibit cell flattening can prevent and/or reverse dedifferentiation. This seems to be the case of microcarrier cultures. For instance, the use of collagens type I microcarriers (collagen) has been reported to support both chondrocyte proliferation and phenotype expression (92). Articular chondrocytes propagated in serial passages (5 passages) as monolayer and re-seeded at passage 6 in collagen microcarriers had no detectable staining for collagen type I and stained intensely for collagen type II. More recently, macroporous gelatin microcarriers (CultiSpher-G) have been also found to be effective matrices for human nasal chondrocyte (93) and bovine chondroprogenitor cells (94) expansion, while maintaining the ability of chondrocyte differentiation. These studies demonstrated that the microcarrier suspension culture system supports growth and enhances expression of the differentiated phenotype. Attachment to a constrained surface and the fluid shear forces on the microcarriers during suspension culture may have helped chondrocytes to reacquire their rounded shape and produce cartilage matrix components. Although the exact mechanism by which chondrocyte redifferentiation is induced through microcarrier expansion has not yet been elucidated, this technique shows promise for cartilage tissue engineering approaches. The maintenance of the chondrocyte phenotype has been also reported when cultured in three-dimensional biodegradable polymer scaffolds to regenerated cartilaginous tissues *in vitro* (95). In this system, the surface chemistry and geometry of the polymer scaffold, (e.g., polyglycolic acid (PGA) mesh) with fiber diameter comparable to that of a chondrocyte, minimize the focal points for cell adhesion and thus prevent cell flattening (28, 95). This ability to support the growth of human chondrocytes and to maintain their original phenotype has been also reported using a hyaluronic-acid-based biodegradable polymer (96). Human chondrocytes, expanded in monolayer cultures for three to four passages and seeded on this material were able to express

and produce collagen type II and aggrecan and downregulate the production of collagen type I.

#### *Growth Factors*

Growth factors can in principle modulate chondrocyte proliferation and differentiation (97). It has been reported that chondrocytes expanded in monolayer for two passages in the presence of fibroblast growth factor-2 (FGF-2) dedifferentiate, but fully maintained their potential for redifferentiation in response to environmental changes (98) and also modulated the subsequent responsiveness of the cells to bone morphogenetic protein-2 (BMP-2). The use of sequential exposure of bovine calf articular chondrocytes to FGF-2 during monolayer expansion and to BMP-2 during three-dimensional culture on PGA scaffolds improved the engineering cartilage tissue (99). In addition, it has been demonstrated the chondrogenesis of human adult articular chondrocytes can be enhanced if they are expanded in the presence of a combination of factors and differentiated with factors belonging to the same superfamily of BMPs (100). Concretely, it has been reported that chondrocytes expanded in monolayer in the presence of FGF-2/TGF- $\beta$  displayed a higher proliferation rate and more dedifferentiation, but also higher capacity to redifferentiate in response to supplementation of serum free medium with TGF- $\beta$  and dexamethasone during three-dimensional cultures. Similar results have been recently reported by culturing bovine chondroprogenitor cells in the presence of TGF- $\beta$ 1 (55). These results evidence that growth factors during chondrocyte expansion not only influence cell proliferation and differentiation, but also the cell potential to redifferentiate and respond to regulatory molecules upon transfer into a three-dimensional environment.

The issue of phenotype expression and differentiation is of vital importance on the success of cartilage tissue engineering applications. All the approaches described above could potentially improve the redifferentiation capacity of autologous chondrocytes isolated from a small biopsy and expanded *in vitro*. Nevertheless, unless the expansion is performed in three-dimensional carriers soon after isolation, it has not yet been demonstrated that extensively expanded chondrocytes can be induced by exogenous factors to regenerate cartilaginous tissue at a rate and to an extent comparable to freshly isolated chondrocytes. Moreover, there are also evidences suggesting that it is increasingly difficult to redifferentiate them in three-dimensional cultures after extensive monolayer expansion (82). The problem resides in the

fact that is precisely extensive expansion what it would be necessary for the development of feasible tissue engineering products in the near future. Whether from allogeneic sources, or from some kind of autologous progenitor cells, the use of alternative sources of cell seems to be mandatory for the success of tissue engineering therapies.

### *Alternative source of cells*

Establishing a reliable source of cells is a principal priority for tissue engineers (101). Cells used in tissue engineering may be drawn from a variety of sources, including primary tissues and cell lines. Primary tissues may be xenogeneic (from different species), allogeneic (from different members of the same species), syngeneic (from a genetically identically individual) or autologous (from the same individual).

#### *Xenogeneic and/or allogeneic*

Although animal cells are a possibility, ensuring that they are safe remains a concern, as does the high likelihood of their rejection by the immune system (102). For those reasons, human cells are favored. Currently, the use of allogeneic cells in the setting of cell-polymer constructs is still limited by the need for host immunosuppression. However, with the advent of techniques to render cells immunologically “transparent”, the use of banked xeno/allogeneic cells may become a clinical reality (101). The creation of cells that could be used as universal donors might for instance be achieved by using molecules that mask the histocompatibility proteins on the cell surface that normally identify the donor cells as non-self (103-105). This type of approach is being explored to make pig cells acceptable for transplantation to patients with Parkinson’s disease (105). In principle, such universal donor cells would not be expected to be rejected by the recipient; they could be generated for various types of cells from many different tissues and kept growing in culture until needed. Nevertheless, it is not yet clear how universal donor cells will perform in large-scale clinical trials (101).

#### *Stem and progenitor cells*

Because of the difficulties exposed before for the different cell sources, the majority of tissue engineering advances to date have employed primary autologous cells (106). In the case of articular cartilage, the limitations of adult chondrocytes to maintain their phenotype expression and differentiation ability after extensive expansion *in vitro* has led to the

investigation of the potential use of pluripotent stem cells and progenitor cells as a source for tissue engineering. The recent identification and isolation of human embryonic stem cells offers one approach to the problem. Cells derived from human embryonic blastocysts can be proliferated through multiple generations and made to differentiate into the appropriate cell type (107). Recent studies on embryonic stem cells involved in the creation of cartilage has shown encouraging results (108, 109), suggesting that in the future these cells may become a potential tool for repairing articular cartilage defects. However, researchers are still a long way from being able to manipulate embryonic stem cells in culture to produce fully differentiated cells that can be used clinically (102). In addition, the use of embryonic stem cells poses major ethical problems that need to be addressed.

A more immediate goal would be to isolate adult stem cell or progenitor cells present in a differentiated tissue. Such progenitors have taken some of the steps toward becoming specialized, but because they are not yet fully differentiated they stay flexible enough to replenish several different cell types. Also, in comparison to the embryonic stem cells, the use of adult stem cells is generally well accepted by the society. Their progeny includes both new stem cells and committed progenitors with a more restricted differentiation potential. These progenitors in turn give rise to differentiated cell types (110). After transplantation some of these cells engraft tissues other than those of their own origin and respond to alternate environmental cues by displaying the phenotypes of such host tissues (111).

This approach has included mesenchymal stem cells, which are capable of differentiating into bone, cartilage, tendon and muscle (112-114). Bone marrow mesenchymal stem cells or stromal cells (BMSCs) are responsible for the maintenance of bone turnover through life and can be regarded as a mesenchymal progenitor/precursor cell population derived from adult stem cells. Cultured BMSCs can be stimulated to differentiate into bone, cartilage, muscle, marrow stroma, tendon, fat and a variety of other connective tissues (115, 116). The harvest of a limited bone marrow sample is an easy and relatively safe procedure. Large numbers of BMSCs can be obtained in culture, making it possible to engineer transplantable constructs composed of these cells in appropriate scaffolds (48). The properties of BMSCs are deeply influenced by the microenvironmental conditions. Culture conditions for BMSC remain essentially the same as the ones originally described by Friedenstein *et al.* (1966). However, culture conditions that allow expansion without loss of differentiation potential are difficult to establish for most adult stem cells (48) and to obtain a large number of chondroprogenitors,

the effects of several growth factors on proliferation and differentiation of BMSC are being investigated (112, 117-119). Although cells potentially useful for connective tissue engineering can be isolated from a variety of tissue, BMSC remain, at the moment, the most interesting and widely accepted cell system to be used for both preclinical and clinical studies of cartilage regeneration and repair (48). Whereas a cartilage biopsy from the joint to obtain differentiated chondrocytes for cell therapy represents an additional injury to the cartilage surface, possibly detrimental to the surrounding healthy articular cartilage, the use of BMSCs avoids this problem altogether. Furthermore, BMSC can be transduced with various viral vectors and are, thus, interesting potential candidates for somatic gene therapy in local or systemic pathologies (120). A critical issue for the future, from a tissue engineering standpoint, is to learn how to control the permanent differentiation of stem cell populations into the desired cell types. There are also a number of technical hurdles such as the need for pure stem cell preparations (i.e., those without other cells such as fibroblast mixed in), methods to reduce cell adhesion during culture, and processes to increase the production of the large numbers of cells needed to create tissue (101).

Alternatively, cells with chondro-osteoprogenitor features have been isolated from several tissues, including periosteum, spleen, thymus, skeletal muscle, adipose tissue, skin, retina and articular cartilage surface (48, 121-127). However, for several reasons such as hardly accessible tissue source, low cell frequency and limited information the use of the majority of these progenitors in tissue engineering has not been always straightforward (48).

One of alternative population of chondroprogenitor cells have been recently identified from the superficial zones of articular cartilages (121). The articular cartilage is not a homogeneous tissue, with biochemical and morphological variations existing from the surface zone to the deeper calcified layer. Consequently, the differentiation and proliferation events occurring during the development of articular cartilage must be strictly controlled both temporally and spatially in order for the distinct zonal architecture of the tissue to be established. Various studies have shown that the surface zone of articular cartilage is centrally involved in the regulation of tissue development and growth. Not only does the surface of articular cartilage play a major role in the morphogenesis of the diarthrodial joint via differential matrix synthesis (128), but also the expression of many growth factors and their receptors at the articular surface (129, 130) suggest that this region represents an important signalling centre. In fact, it has been shown *in vivo* that the surface zone of the

articular cartilage is responsible for the appositional growth of articular cartilage (130). For such a mechanism to occur, a population of stem/progenitor cells must reside within the articular cartilage to provide transit amplifying progeny for growth. In that way, new progeny are generated at the articular surface, expand within the transitional zone and progress to terminal differentiation in the lower zone (130). Recently a number of studies have corroborated this hypothesis by the identification and partially characterization of a population of cells isolated from the superficial zone of the articular cartilage (55, 121, 131-133). These characteristics have included a prolonged cell cycle time at the articular surface (130), differential adhesion to fibronectin, differential integrin and Notch family expression and an ability to form large numbers of colonies *in vitro* from a low seeding (131, 132), properties that are common to known progenitor cell populations of other tissues (134).

Additionally, this population of cells exhibits a significant degree of plasticity in its differentiation pathways (121), another requisite of any stem cell population (135). The engraftment of bovine surface zone-derived cells in an embryonic chick tracking system resulted in the formation of a variety of connective tissue types including bone, tendon and perimysium (121). Moreover, the chondrogenic ability of this cell population has also been proved *in vitro*. Using a specific medium similar to that reported for the induction of chondrogenesis with mesenchymal stem cells, Martin *et al.* showed that this population of superficial zone cells were able to synthesise hyaline cartilage matrix when cultured in three-dimensional pellets (55). Further studies have also demonstrated the high expansion potential of these chondroprogenitor cells in comparison to normal bovine chondrocytes (133). The cells retained the ability to synthesise a cartilage-like hyaline matrix rich in glycosaminoglycans and collagen type II even after 11 passages which equated to 25 population doublings. On the other hand, normal chondrocytes isolated from bovine articular cartilages rapidly dedifferentiated in monolayer and had completely lost the ability to redifferentiate in pellet culture after 8 passages (or 13 population doublings), although this loss of redifferentiation potential could have occurred at an earlier stage of expansion as suggested by other authors (136, 137). The enhanced potential of these articular cartilage progenitor cells to retain the ability to form cartilage after extensive expansion in culture could constitute a major step forward for cartilage repair as it may enable the generation of large cell banks for use in future allogeneic tissue engineering applications. Investigations are currently focusing on employing these cells in cartilage repair strategies. It seems that the undifferentiated nature of these progenitor cells could be used to reproduce the structural and

hence biochemical properties of normal articular cartilage and thus integrate more fully into articular cartilage lesions. On this regard, it has been recently shown in a bovine model that a transplanted chondroprogenitor enriched population performs better at filling a partial depth lesion in a short period of time than a typical population of chondrocytes taken from the full thickness of articular cartilages (138).

### ***Large scale culture systems for expansion of chondrocytes***

Currently available culture systems for cell expansion are described below. The advantages and disadvantages of these systems are summarized in Table 4.

**Table 4. Selected culture systems for anchorage-dependent cells**

<b>Culture System</b>	<b>Advantages</b>	<b>Disadvantages</b>
T-Flask	Simple Well characterized	Difficult to scale-up Labour intensive No continuous control of culture parameters No perfusion of culture medium
Cell Factories	Large scale (relative to T-flasks)	Still labour intensive in large scale No continuous control of culture parameters No perfusion of culture medium
Roller Bottle	Large surface area to volume ratio (relative to T-flask)	Still labour intensive in large scale No continuous control of culture parameters No perfusion of culture medium
Cell-Cube <sup>TM</sup>	Large surface area to volume ratio (relative to T-flask) Perfusion of culture medium	Expensive Still labour intensive in large scale No continuous control of culture parameters
Pellet cultures	Simple Perfusion of culture medium Culture parameters can be continuously controlled	Cell harvesting Reduced proliferation rates
Hydrogel cultures	Perfusion of culture medium Culture parameters can be continuously controlled	Mass transfer limitation may occur Reduced proliferation rates
Microcarrier cultures	Greatest surface area to volume ratio Perfusion of culture medium Culture parameters can be continuously controlled Well documented technology	Cell exposed to shear forces Some cell types show reduced growth compared to monolayer culture

### ***Monolayer culture***

Expansion of anchorage-dependent cells, including primary chondrocytes or chondroprogenitor cells, largely rely on simple monolayer culture flasks (i.e., T Flasks, Petri dishes or tissue culture well plates) to date. However, if large scale operations are to be considered, alternatives to these traditional tissue culture flasks are mandatory. There are currently available several alternatives of large scale culture vessels and systems from different manufacturers. One of these alternatives is based on the roller bottle technology. The concept of growing cells as rotating cultures was originally developed at Johns Hopkins University (139) as a mean of growing larger quantities of anchorage-dependent cells. Since then, much larger glass roller bottles have been in common use for growing large numbers of cells, especially for viral vaccine production (140, 141). Besides providing larger surface areas for growth, this culture technique may also have two additional advantages over traditional static monolayer cultures: first, its gentle agitation prevents gradients from forming within the medium that may adversely affect growth; second, cells spend most of their time covered by only a thin layer of medium allowing superior gas exchange. The RollerCell 40 (CELLON S.A., Luxembourg) is a self-contained, automated roller bottle processing system, and its use has been recently reported for large scale production of retroviral vectors (142). Up to ten RollerCell 40 units can be linked together in series with a single CPU (RollerCell Max; CELLON S.A., Luxembourg), providing a surface area of  $3.5 \times 10^5 \text{ cm}^2$  (equivalent to 200 roller bottles of  $1750 \text{ cm}^2$ ). An alternative to roller bottles is the Corning CellCube System (Corning Incorporated, USA), an integrated modular bioreactor system that has been successfully used in a variety of large scale culture applications (142-146). This system allows continuous perfusion of fresh media and can be scaled up to  $3.4 \times 10^5 \text{ cm}^2$  of growth surface using the same control package. Finally, if the choice of static systems is still preferred, multitray battery systems would be recommended. This system is designed for large scale operations, and its use has been reported in a variety of culture processes, including the production of human fibroblast interferon (147, 148). Two commercially available multitray systems are the Corning CellSTACK (Corning Incorporated, USA) and the Nunclon<sup>TM</sup>  $\Delta$  Cell Factory (Nunc, Denmark). Both products allow assembling up to 40 trays, providing a surface area of  $2.5 \times 10^4 \text{ cm}^2$ .

Despite the improvements introduced by the use of large scale operation units, the main problem with monolayer systems reside in their low ratios of surface to volume. A flat

monolayer configuration is the most straightforward way of providing the surface for the cells to attach, but not necessarily the most efficient in term of scalability. Due to this limitation, optimization of the culture conditions for the expansion of the cell population in monolayer constitutes an essential step in the process of tissue engineering of articular cartilage. Melero-Martin *et al.* have recently illustrated the importance of culture optimization using chondroprogenitor cells in monolayer (149). The analysis of both seeding density and passage length was revealed crucial to establish optimal culture conditions for monolayer expansion. The authors reported that the determination of the optimal seeding density and the corresponding passage length for cell expansion in a serial passaging operation was a compromise between growth kinetics and process time (149). Additional considerations concerning the running cost of the process were introduced: although the optimal passage length gave the desired expansion factor in a minimum process time, the selection of an alternative value of passage length was shown to reduce the cost of the expansion process in more than 60%. Nevertheless, if production is to be significantly increased, the number of culture units has to be remarkably increased making the process time consuming and laborious. The result is that the process may not be cost effective. Although the large scale alternative systems impose a notorious improvement when compared to simple T-Flasks, their inherent limitations in term of scalability could finally compromise the suitability of these systems in very large scale operation.

### *Three-dimensional expansion methods*

Three-dimensional systems could potentially provide the improved ratio of surface to volume necessary to cope with the scale of cell expansion require for allogeneic tissue engineering applications.

#### *Pellet culture*

Since most primary anchorage-dependent cells in suspension are known to form aggregates, these aggregates could be grown in suspension cultures that promote the multiplication of the cells in the aggregates. For example, articular chondrocytes has been maintained in high-density aggregated cultures (74-77) that support cell proliferation while maintaining the chondrocyte phenotype (78, 79). It has been also reported for that an appropriate mixing regime could promote controlled cell aggregation in suspension by maintaining aggregate diameters in a relatively narrow range of 11-32  $\mu\text{m}$  (which corresponded to 1-16 cells) (28).

However the strong interactions cell-cell generated during this kind of culture make the harvesting of viable chondrocytes difficult to achieve.

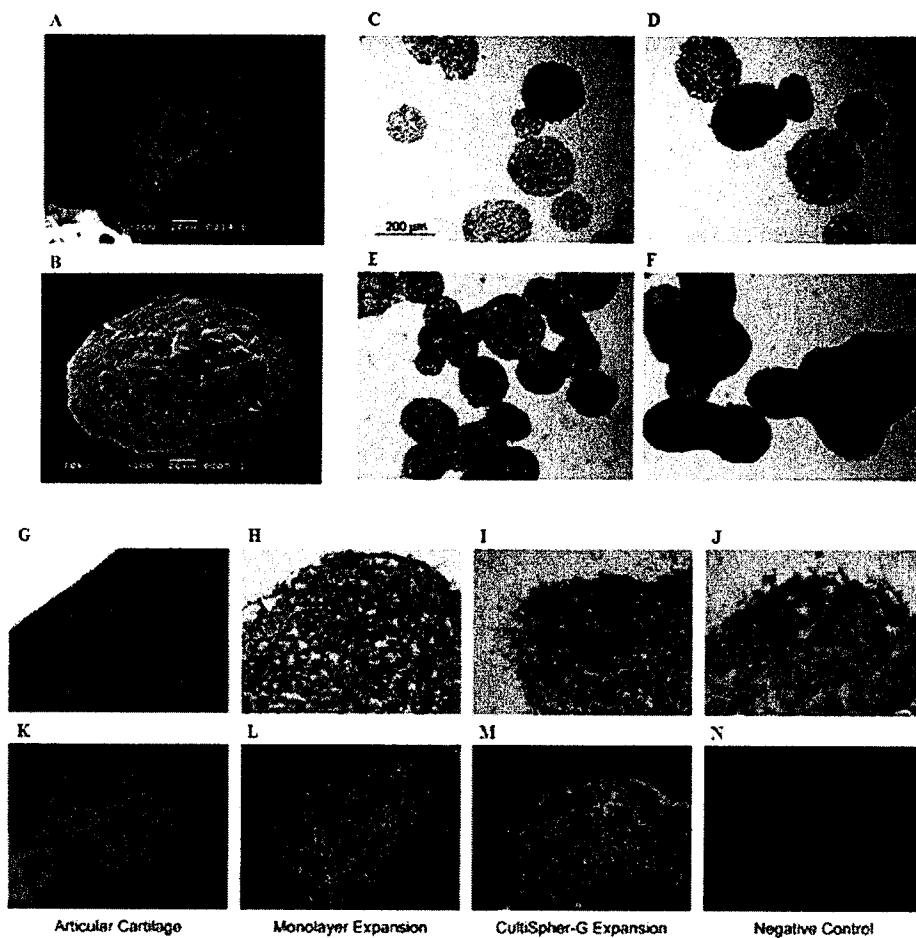
#### *Encapsulation inside hydrogel beads*

Cells can be trapped inside hydrogel beads such as agarose, collagen and alginate and then introduced into bioreactors and culture in suspension. Since these kind of three-dimensional culture systems are known to prevent/restore articular chondrocyte differentiation, the number of reported studies using these methodology is extensive (60, 80-88, 150). One of the most studied is the alginate system. During alginate culture chondrocytes divide to form cell clusters and also synthesize cartilage-specific matrix components which form a halo around the cells (151). While cell number increase during initial culture, a plateau is reached at a later time indicating an inhibition of cell proliferation (86). Consequently, with this kind of culture techniques the recovery of viable cells for passage is necessary. Individual cells can be recovered by dissolving the alginate beads, using calcium chelating agents followed by enzymatic digestion to remove elaborated matrix, allowing repeat passage to be performed. Although there are little doubts about the chondrocytic phenotype enhancement produced by this kind of cultures, the expansion potential achievable with repeat passage in alginate beads it is less clear. While early studies have indicated that the rate of chondrocyte proliferation is greater in monolayer than these three-dimensional culture systems (152), the same authors claim that the rate of expansion in alginate would still be acceptable for practical autologous chondrocyte transplantations (150). However, for potential allogeneic applications (where the expansion demand would be far higher than for autologous applications), the suitability of such proliferation rate for the development of large scale cell expansion processes would need to be re-examined.

#### *Microcarriers*

Microcarriers are bead-like structures ranging from 100-200  $\mu\text{m}$  in diameter that can be held in homogeneous suspensions in stirred bioreactors. In microcarrier cell culture technology anchorage-dependent animal cells are grown on the surface of small spheres which are maintained in stirred suspension culture. Cells attach and spread on the surface provided by the microcarriers and gradually grow into confluent layers. This technology is replacing conventional monolayer cell culture methods since the extremely high surface area to volume ratio afforded by microcarriers suppose a significant improvement for process scale-up. Apart

from this enhanced scalability, microcarrier cells culture technology allows efficient monitoring and culture control while maintains reduced processing costs and reduced risk of contamination (153, 154). Commercially available microcarriers (including porous and non-porous) have been made from a variety of materials such as DEAE Sephadex, collagen, glass and gelatine and their applications have included an extensive diversity of cells. For example, in the context of articular cartilage, human chondrocytes have been reported to proliferate and produce matrix components in microcarrier suspension culture by using both Cytodex-3 (denatured collagen coated. Amersham pharmacia biotech, Sweden) and Collagen (collagen type I derived beads. ICN, US) non-porous microcarriers (92, 155, 156). Alternatively to non-porous microcarriers, macroporous microcarriers provides interior surface for cell attachment protecting the cells against the detrimental hydrodynamic forces (157, 158) and potentially improving the yield of expanded cells. One example of porous microcarriers is CultiSpher-G (Percell Biolytica, Sweden). CultiSpher-G is made of gelatine that is derived from collagen, simulating the *in vivo* environment more closely than with other commercially available microcarriers. One major advantages of CultiSpher-G over other macroporous microcarriers is that its gelatine matrix can be dissolved with a variety of proteolytic enzymes (such as dispase and trypsin) and consequently cell harvesting is facilitated and the need to separate cells and microcarriers eliminated. The use of CultiSpher-G have been already reported for a variety of animal cell including human nasal chondrocytes (93) and bovine chondroprogenitor cells (94). Figure 2 illustrates the expansion suitability of chondroprogenitor cells isolated from the superficial zone of bovine articular cartilages using macroporous microcarrier in spinner flask cultures. In addition, during microcarrier cultures, cells are capable of undergoing bead-to-bead migration (94), which allow subcultivation to be performed without a harvesting step, thus improving the scalability of the expansion process. Serial subcultivation of cells on microcarriers is one of the most cost-effective means of establishing animal cell culture production facilities and can be achieved using a series of culture vessels of increasing volume and capacity (154). The cells cultured in one vessel can be subsequently used to inoculate the following large vessel. This approach to scale-up would eliminate the large numbers of culture vessels, such as roller bottles or multi-trays that would be otherwise required to obtain sufficient cells. By employing microcarrier cultures it will be possible to obtain large number of cells for cartilage tissue engineering applications. Not only its satisfactory expansion potential, but more importantly the cost and operational advantages over traditional monolayer culture make this system a feasible alternative method for the extensive expansion of chondroprogenitor cells. In addition, large scale microcarrier culture



**Fig. 2.** Expansion of chondroprogenitor cells on macroporous microcarriers. Chondroprogenitor cells are able to attach and proliferate until confluence on macroporous microcarriers. (A) SEM micrographs of empty CultiSpher-G microcarriers and (B) confluent microcarriers after 7 day of culture. In addition, chondroprogenitor cells are capable to undergoing bead-to-bead migration, which allowed subcultivation to be performed without a harvesting step, thus improving the scalability of the expansion process. Panels C-F were taken from samples of spinner flasks that were seeded using as the inoculum cell-loaded CultiSpher-G microcarriers from a previous culture. Light micrographs of the cultured microcarriers were visualised at day 2 (C), day 4 (D), day 6 (E) and day 8 (F) after MTT staining. Finally, the expanded cell population was proven to maintain the ability to undergo chondrogenesis *in vitro*, an essential requisite for any proposed expansion method. Chondroprogenitor cells expanded either in monolayer or CultiSpher-G were subsequently cultured as pellets for 12 days for chondrogenesis induction. Histological sections of (G) bovine articular cartilage, (H) monolayer expanded cell pellet and (I) CultiSpher-G expanded cell pellet after safranin-O staining for GAG visualisation. Immunohistochemical sections of (K) bovine articular cartilage, (L) monolayer expanded cell pellet and (M) CultiSpher-G expanded cell pellet after incubation with monoclonal antibodies for collagen type II visualisation. (J and N) Negative controls obtained in the absence of TGF $\beta$ 1 during the pellet culture period. This figure was adapted from reference (94) with permission.

is a very well documented and accepted technology. Industrial scale microcarrier applications have been proved to be reliable and cost-effective for the manufacture of both human and animal health care products. Virus vaccines, interferons, plasminogen activators and urokinases, cytokines, hormones and a variety of factors such as platelet derived growth factors (PDGF), epithelial growth factor (EGF), tumor necrosis factor (TNF), erythropoietin (EPO), colony stimulating factor (CSF) and others are currently produced from such microcarrier facilities (154). Some of these products are already available on the market as human and animal diagnostics and therapeutics. Many are undergoing evaluation and clinical trials and even more are in the developmental phase.

### **Conclusion**

A continuous production of large scale quantities of chondrocytes will be shortly required for the manufacture of engineered cartilages, especially if allogeneic tissue engineering products are to be used for the treatment of cartilage defects. However, routine tissue culturing methodologies can hardly cope with the scale of cell production that would eventually be required. In this review we have addressed two of the major constraints for the successful production of large number of articular chondrocytes: the necessity of alternative sources of stem/progenitor cells and the development of feasible large scale cell expansion processes.

In the process of cartilage tissue engineering, it is important to ensure that the expanded cell population retains its phenotypic function. Chondrocytes derived from articular cartilage biopsies have only a limited proliferative potential. They dedifferentiate upon repeated passaging and the number of cell divisions chondrocytes undergo *in vitro* decreases with age. The issue of phenotype expression and differentiation has led to the investigation of the potential use of stem/progenitor cells as a source for tissue engineering. These have included mesenchymal stem cells, which are capable of differentiating into bone, cartilage, tendon and muscle. More recently, a new population of chondroprogenitor cells isolated from the superficial zone of the articular cartilage has been identified and partially characterized. Chondroprogenitor cells are able to differentiate and produce cartilage-like extracellular matrix upon extensive expansion *in vitro*, providing a promising alternative source of cells. This enhanced expansion potential of chondroprogenitor cells together with their ability to form cartilage after extensive culturing could constitute a step forward for cartilage repair as it may enable the generation of large cell banks for use in tissue engineering applications.

The second fundamental issue addressed in this review is the necessity of feasible large scale expansion protocols. Despite the improvements introduced by the use of large scale operation units, monolayer systems present very low ratios of surface to volume, which inevitably make them inefficient in term of scalability. If production of articular chondrocytes is to be significantly increased, the number of culture units has to be remarkably increased, making the process time consuming and laborious. The result is that the expansion process may not be cost effective. The introduction of three-dimensional alternative systems could potentially provide the improved ratio of surface to volume necessary to cope with the scale of cell expansion required for allogeneic tissue engineering applications. One of these alternatives is the use of cell-seeded microcarriers for cell expansion. Both mature chondrocytes as well as chondroprogenitor cells have been already shown to growth satisfactorily in a diversity of microcarrier materials. Additionally, chondroprogenitor cells were shown to be capable of undergoing bead-to-bead migration, which allow subcultivation to be performed without a harvesting step, thus improving the scalability of the process. Finally, the microcarrier-expanded cell population has been also proven to maintain the ability to undergo chondrogenesis *in vitro*, an essential requisite for any proposed expansion method. Therefore, not only due to its satisfactory expansion potential, but more importantly the operational advantages over traditional monolayer cultures make microcarrier cultures a feasible alternative method for extensive cell expansion.

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